# The Translational Repressor Pumilio Regulates Presynaptic Morphology and Controls Postsynaptic Accumulation of Translation Factor eIF-4E

**Kaushiki P. Menon, Subhabrata Sanyal, Yasuaki Habara, Ricardo Sanchez, Robin P. Wharton, Mani Ramaswami and Kai Zinn**

## Supplemental Experimental Procedures

### Genetics: Lethality Induced by GAL4-Driven Pum Expression

The full-length cDNA constructs either had the normal *pum* 3′UTR or a 3′UTR (*tub*) from the α-*tub84B* mRNA that appears to increase expression when incorporated into several different chimeric mRNAs (R.P.W, unpublished data). When UAS-full-length Pum was crossed to Elav-GAL4 at 29°C in a wild-type background, most larvae did not survive to third instar. Escaper third instar larvae were observed in this cross, but none were seen when the UAS-full-length Pum-*tub* 3′UTR line was used. At 18°C, no lethality was observed in crosses with either *pum* construct. GAL4 transactivation is more efficient at high temperatures, so these results imply that lethality is correlated with the level of expression of full-length Pum. The UAS-Pum RBD construct did not cause lethality when expressed in neurons at any temperature.

Muscle overexpression of full-length Pum in a wild-type background was also toxic, and UAS-full-length Pum larvae bearing the strong muscle drivers 24B-GAL4 or G14-GAL4 died prior to third instar at all temperatures. To test rescue by Pum expression in muscles, we thus used a genetic background in which an attenuated steroid-inducible GAL4 (GS-GAL4) was expressed in muscle via a myosin heavy chain (MHC) promoter (Osterwalder et al., 2001; Roman et al., 2001). Steroid (RU486) induction of GS-GAL4 in hatched *F1* larvae carrying both (MHC)-GS-GAL4 and UAS-full-length Pum transgenes with either 3′UTR resulted in lethality at all temperatures. However, MHC-GS-GAL4 drives late steroid-independent gene expression in larval muscles (Osterwalder et al., 2001), and we found that these *F1* larvae did survive in the absence of steroid. Pum is expressed by this driver in the absence of steroid (as checked by immunohistochemistry with Pum antibody; data not shown), and this "leaky" muscle expression can rescue the postsynaptic *pum* phenotypes. We also found an MHC-GAL4 insertion that allowed survival of larvae expressing full-length Pum-*tub* 3′UTR and used this to drive Pum expression in the genetic interaction experiment of Figure 5C.

### Quantitation of Pum Protein at the NMJ

Wild-type and mutant larval fillets that were double stained with Pum and Dlg antibodies were imaged at the same time with identical settings on the confocal microscope. Images were quantified with Image J software (NIH) by drawing the region of interest on the Dlg panel to prevent bias. Since both Pum and Dlg form concentric circles around the edge of the bouton, the region that was outlined for intensity measurements was at the border of the Dlg ring for each bouton. The average gray value per pixel within the selection (mean) was obtained for both Dlg and Pum channels, and the ratio of the mean intensity per pixel of Pum and Dlg was determined.

### Quantitation of Synaptic Phenotypes

For all genotypes (wild-type, mutant, and rescued larvae), crosses were set up at 18°C, and third instar larvae were taken from the culture medium. Since *pum* mutant larvae generally stayed at the bottom of the vial in culture medium, wild-type, *pum* larvae, and rescued larvae were always also taken from the culture medium and not from the wall of the vial. To obtain an accurate measurement of bouton size, larvae were put in phosphate-buffered saline and heated for 45 s at 62°C to stretch the muscles. They were then dissected and stained with primary and secondary antibodies.

Bouton size was measured as follows: using Zeiss software, we measured the areas of three terminal boutons from each Type Ib branch in 1D4/Dlg-stained preparations and then determined the average size. Type Ib boutons on muscle 12 were defined by Dlg staining (Ib boutons display stronger Dlg staining than type Is boutons). The number of type Ib boutons was quantified from similarly stained preparations. We also evaluated muscle size in all the genotypes analyzed in this paper, and none of the genotypes showed any significant change in larval muscle areas relative to controls.

### Induction of Movement and Quantitation of eIF-4E

For all genotypes (wild-type, mutant, and rescued larvae), third instar larvae were taken from vials in which the food had the consistency of a liquid slurry, so that larval motility was minimal. These crosses were set up at 18°C with about ten virgins per vial, so that they contained a high density of larvae; this facilitated liquefaction of the food. Larvae were then placed on grape juice agar plates (without yeast) for 3 hr at 29°C to induce movement (Sigrist et al., 2003). They were then dissected in ice-cold fix (4% paraformaldehyde) for 5 min and incubated in eIF-4E antibody (dilution 1:500) overnight.

Quantitation of the amount of eIF-4E in NMJ aggregates was performed using Image J software (NIH). The area (square pixels) and the mean gray value per pixel of each aggregate were obtained, and the total amount of eIF-4E in aggregates at each NMJ was then summed.

Two "wild-type" strains were used as controls in the eIF-4E experiments, in order to ensure that the amounts of eIF-4E in *pum* mutants were increased relative to both reference strains. In NMJs of $w^{1118}$ (*Canton S*-derived), an average of 2.1 aggregates were observed per NMJ after transfer, consistent with the numbers reported for another *w−* strain (Sigrist et al., 2000, 2003). *Oregon R* (*OR*) larvae had an average of 4.75 aggregates per NMJ; Figure 6A). Quantitation of the areas and intensities of these aggregates indicated that *OR* larvae had about 2-fold more synaptic eIF-4E per NMJ than do $w^{1118}$ larvae (344 versus 169 in arbitrary fluorescence units; Figure 6B). This difference largely reflects a larger fraction of hemisegments in $w^{1118}$ that had no aggregates. When only NMJs with aggregates were included in the analysis, the levels of eIF-4E at the NMJ were almost identical in the two genotypes (380 for $w^{1118}$ versus 399 for *OR*). For experiments in which eIF-4E aggregates were quantitated in less motile larvae maintained in liquid food, we took larvae directly from the culture vial and stained these alongside larvae from the same vial that had been transferred to grape juice agar plates. In this experiment, the levels of eIF-4E in aggregates in $pum^{ET9}/pum^{ET7}$mutants were 1499 in liquid (n = 32) versus 2327 in larvae transferred to solid media. In either of the two wild-type reference strains, we observed very few segments with eIF-4E aggregates in larvae not transferred to solid media, so we did not attempt to quantitate eIF-4E levels in these larvae.

### Data for eIF-4E Staining Results Displayed in the Bar Graphs of Figure 5

In Figure 5A, the bar for $pum^{ET9}/pum^{ET7}$ (et9/et7) pools the numbers obtained for three sets of larvae, since their counts were not significantly different from each other: (1) $pum^{ET9}/pum^{ET7}$ larvae from a cross between the original mutant alleles $pum^{ET9}$ and $pum^{ET7}$ (actual aggregate count = 24.0 ± 3.0; n = 24 NMJs); (2) *GSG,*$pum^{ET9}/pum^{ET7}$ larvae from a cross of recombined line *GSG,*$pum^{ET9}$ (GSG is the muscle GAL4 driver, MHC-GSGAL4) with $pum^{ET7}$ (count = 24.5 ± 2.9; n = 28 NMJs); and (3) *UAS-pum-tub3′UTR,*$pum^{ET7}/pum^{ET7}$ larvae from a cross of $pum^{ET9}$ with recombined line *UAS-pum-tub3′UTR,*$pum^{ET7}$ (count = 26.4 ± 2.5; n = 28 NMJs). The latter two sets of larvae are controls for the muscle rescue experiment. The numbers of NMJs scored for

the different genotypes in graphs 5A and 5B are as follows: $w^{1118}$, n = 18; *OR*, n = 44; *et9/+*, n = 24; *et9/et7*, n = 80 (pool of three different lines); *msc/et7*, n = 7; neuronal rescue, n = 17; muscle rescue, n = 41.

In Figure 5B, the values for the three different genotypes pooled to generate the et9/et7 bar are as follows: 2327 ± 416 (n = 24 NMJs), 2140 ± 393 (n = 28 NMJs), 1790 ± 228 (n = 28 NMJs).

In Figure 5C, the et9/et7 bar pools the Is bouton counts from three genotypes: $pum^{ET9}/pum^{ET7}$, *GSG,*$pum^{ET9}/pum^{ET7}$, and $pum^{ET9}$/*UAS-pum,*$pum^{ET7}$ (muscle rescue controls; see legend for Figure 5A above), since these were not significantly different from each other. Muscle rescue and neuronal rescue genotypes are as in Figure 5A.

**Conditions for GluRIIa Staining**

Larvae were taken from crosses set up at 18°C as above and dissected directly after removal from liquid slurry food. These larvae were dissected live in HL-3 Ringer's solution and incubated with primary antibody overnight.

**Western Blotting**

Body wall lysates were made from dissected wild-type (wt) and $pum^{ET9}/pum^{ET7}$ ($pum^{-}$ et9/et7) larvae. Lysates were boiled at 95°C for 5 min and loaded on a 7.5% acrylamide gel. Western blotting was performed with rabbit anti-PumN primary (1:5000) and HRP-conjugated donkey anti-rabbit secondary antibodies (1:15,000). For chemiluminescent detection, the ECL Plus kit from Amersham Bio-Sciences was used as per the manufacturer's instructions.

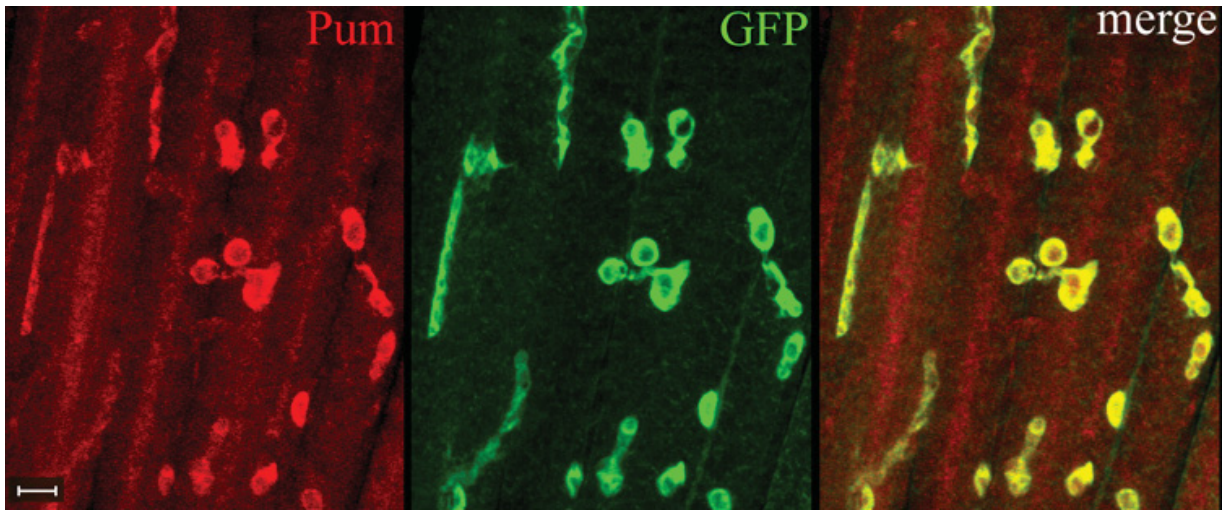


Figure S1. Pum Is Localized to NMJs on Adult Muscles

Abdominal fillets from otherwise wild-type flies bearing a muscle-expressed MHC-*CD8-Sh-GFP* insertion were stained with anti-PumRBD (red) and anti-GFP (green) and visualized with confocal microscopy. Note that all NMJs in the merge panel are yellow, indicating that Pum is localized to the same region as the postsynaptic GFP.

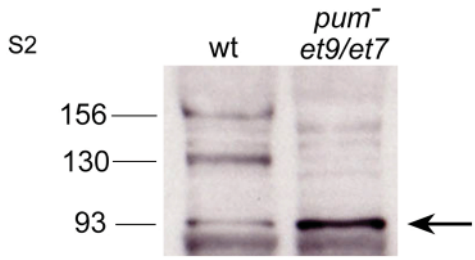


Figure S2. Pum Proteins in Wild-Type and Mutant Larvae

A Western blot of body wall lysate from wild-type larvae ($w^{1118}$; labeled as wt) with anti-PumN shows three major bands whose mobility is consistent with the 156, 130, and 93 kDa species previously seen by others in adult heads and ovaries (Parisi and Lin, 1999; Schweers et al., 2002). The fuzzy band below the 93 kDa species is present with the same intensity for all genotypes and is probably not a Pum protein. In $pum^{ET9}/pum^{ET7}$ body wall lysates ($pum^{-}$ *et9/et7*), the two upper bands are absent or greatly reduced in intensity relative to wild-type, while the intensity of band(s) migrating at about 93 kDa is increased (arrow). Equal amounts of protein were loaded in each lane. In $pum^{ET7}$/*Df(3R)BSC24* mutants, the upper bands are also weak, the 93 kDa band is similar in intensity to wild-type, and an additional lower molecular weight band is observed (data not shown).